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A Low-pH-Inducible, Stationary-Phase Acid Tolerance Response in *Salmonella typhimurium*

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Acid is an important environmental condition encountered by *Salmonella typhimurium* during its pathogenesis. Our studies have shown that the organism can actively adapt to survive potentially lethal acid exposures by way of at least three possibly overlapping systems. The first is a two-stage system induced in response to low pH by logarithmic-phase cells called the log-phase acid tolerance response (ATR). It involves a major molecular realignment of the cell including the induction of over 40 proteins. The present data reveal that two additional systems of acid resistance occur in stationary-phase cells. One is a pH-dependent system distinct from log-phase ATR called stationary-phase ATR. It was shown to provide a higher level of acid resistance than log-phase ATR but involved the synthesis of fewer proteins. Maximum induction of stationary-phase ATR occurred at pH 4.3. A third system of acid resistance is not induced by low pH but appears to be part of a general stress resistance induced by stationary phase. This last system requires the alternative sigma factor, RpoS. Regulation of log-phase ATR and stationary-phase ATR remains RpoS independent. Although the three systems are for the most part distinct from each other, together they afford maximum acid resistance for *S. typhimurium*.

Many bacteria normally considered neutralophilic must survive intermittent exposures to potentially lethal acid conditions that can occur in the natural environment or, if the organism is a pathogen, within a host environment. A resurging interest in how cells cope with low-pH stress has developed partly on the basis of the predicted importance of acid resistance for the successful pathogen (13, 14). *Salmonella typhimurium* is a pathogenic microorganism that produces a typhoid-like fever in mice and as such serves as a useful paradigm in the study of bacterial pathogenesis. As a waterborne and food-borne organism, it encounters low pH in both natural and host environments (5, 18). Previous studies using logarithmically growing cells have shown that *S. typhimurium* can mount a two-staged protective response to low pH (8, 10). The log-phase acid tolerance response (log-phase ATR) includes a pre-acid shock stage in which cells exposed to mild acid (pH 5.8) induce an ATR-specific pH homeostasis system that can function where housekeeping homeostasis systems fail (9). The second stage, termed post-acid shock, occurs as cells are shifted to moderately acid pH levels (<pH 4.5) and involves the induction of over 40 acid shock proteins (ASPs). One or more of these ASPs are essential for acid tolerance development (7).

Although salmonellae exhibit log-phase ATR, they are not always growing logarithmically when they encounter low pH. In the natural environment, they may be present in dilute nutrient solutions and as such will experience starvation-induced stationary-phase conditions. Likewise, in the host environment these organisms rarely if ever experience the optimal growth conditions encountered in the laboratory. Indeed, growth rates of 10 to 20 h are typical in the intestine and intracellular environments (1, 15, 19, 20). With this in mind, we have examined the acid tolerance capability of stationary-phase *S. typhimurium*. We find that stationary-phase cells exhibit a low-pH-inducible ATR (stationary-phase ATR) that is distinct from the log-phase ATR and the RpoS-dependent general stress resistance associated with stationary-phase cells (16).

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MATERIALS AND METHODS

Bacterial strains and cultural conditions. The bacterial strains used throughout this study were all derivatives of *S. typhimurium* LT2. The *atp*::Tn*10* (JF1923) and *fur* (JF2023) mutant strains were described earlier (9, 10). The *rpoS*::Ap^r mutation was kindly provided by S. Libby (4). It was transferred to SL1344 (*rpsL hisG xyl*), an *rpoS*$^+$ *S. typhimurium* strain. The *rpoS*$^+$ phenotype was characterized as the production of copious amounts of catalase where *rpoS* mutants produced no catalase. Culture media included minimal E glucose (24) and Luria-Bertani (17). Antibiotics were added as follows: ampicillin, 60 μg/ml, and chloramphenicol, 40 μg/ml. Incubations were performed at 37°C under semianaerobic conditions (3 ml of medium in test tubes [10 by 100 mm], with shaking at 240 rpm).

Acid tolerance. Stationary-phase ATR was measured by growing cells overnight semianaerobically in a minimal glucose medium at pH 8 (unadapted) and pH 5.5 (adapted). Final pH values were about 7.2 and 4.2, respectively. A total of 500 μl of each culture was centrifuged for 2.5 min (13,000 × *g*), washed in an equal volume of pH 3.0 minimal medium, recentrifuged, and finally resuspended in pH 3.0 medium to 2 × 10^8 to 5 × 10^8 cells per ml (3 ml). Growth-phase-dependent differences in cell size can lead to discrepancies between optical density and viable count measurements. Therefore, care was taken to determine viable counts for each culture by basing percent survival on cell number at time zero. At timed intervals, aliquots were collected and viable counts were determined. Alternatively, cells from 500 μl of a pH 8.0 overnight culture were collected as described above, washed in 500 μl of pH 4.3 medium, and finally resuspended in pH 4.3 medium to 2 × 10^8 to 5 × 10^8 cells per ml. The pH 4.3 treatment continued for 2 h, at which point cells were collected, washed in 500 μl of pH

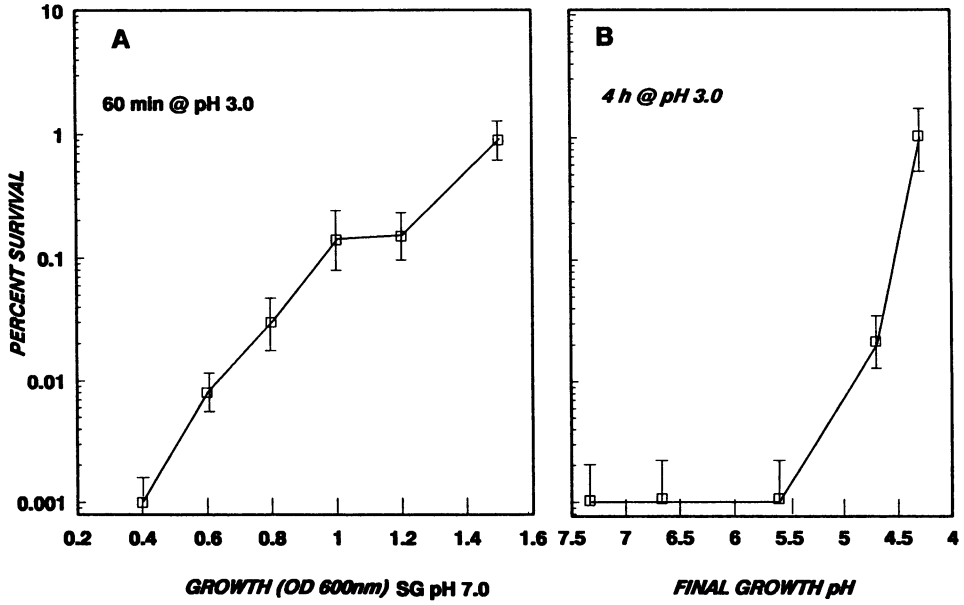

FIG. 1. Acid tolerance of stationary-phase cells. (A) Comparison between log-phase and stationary-phase cultures of LT2. Cells were grown in minimal glucose medium (pH 7.0) to the optical densities indicated on the abscissa. Under these conditions, the transition to stationary phase occurred by optical density at 600 nm of 1.0 with a subsequent slight increase in optical density to 1.5. At each cell density tested, cells were harvested as indicated in Materials and Methods and resuspended at pH 3.0 to between 2×10^8 and 5×10^8 cells per ml. Viable counts were determined at timed intervals during the severe acid challenge, but only that for 60 min is shown. (B) Inducing external pH for stationary-phase ATR. Cells were grown overnight in minimal glucose at various pH values. The final pH values for each culture are shown on the abscissa. Each stationary-phase culture was subjected to severe acid challenge (pH 3.0) as detailed in Materials and Methods. Viable counts were determined at timed intervals, but only the 4-h time results are shown. Error bars indicate range from the mean. SG is E salts glucose medium.

3.0 medium, and finally resuspended in pH 3.0 medium to 2×10^8 to 5×10^8 cells per ml. Again, aliquots were removed for viable counts at timed intervals. It is important to note that these cells will not grow at pH values below 4.5. Log-phase cells were prepared by diluting (1:100) an overnight culture into fresh medium (10^6 cells per ml) and growing it under semianaerobic conditions to 10^8 cells per ml. The log-phase ATR was measured as described previously (6, 8). The buffering conditions of E medium were sufficient to indefinitely maintain constant pH at values below 4.5.

Two-dimensional SDS-PAGE. The modified method for O'Farrell two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was as previously described (22). The first dimension was a pH 5 to 7 (right-to-left) isoelectric focusing gel containing 1.6% (pH 5 to 7) and 0.4% (pH 3 to 10) ampholytes (Bio-Rad). The second dimension was an SDS–11.5% polyacrylamide gel. Figures shown are representative of duplicate gels of two independent experiments.

RESULTS

Stationary-phase cells are more acid tolerant than log-phase cells. To determine the relative acid tolerance of log-phase versus stationary-phase cells, *S. typhimurium* LT2 was grown in minimal glucose medium (pH 7.0) to a variety of cell densities, rapidly collected, and then resuspended in fresh minimal glucose medium at pH 3.0. The suspensions were made to equivalent cell densities (2×10^8 to 5×10^8 cells per ml) for lethal pH challenge. The results revealed that stationary-phase cells were 1,000-fold more acid tolerant than log-phase cells after 1 h of pH 3.0 exposure (1% versus 0.001% survival) and that the tolerance progressively increased with increasing cell density (Fig. 1A).

Demonstration of a low-pH-inducible stationary-phase ATR. Even though cells grown to stationary phase at pH 7.0 were more acid resistant than log-phase cells, these same stationary-phase cells would completely succumb to pH 3.0 by 4 h. Since log-phase ATR required induction by a moderately low pH, we wondered whether the same might be true for a stationary-phase ATR that would enhance stationary-phase acid resistance. Figure 1B reveals that there is a low-pH-inducible stationary-phase ATR. In this experiment, a series of overnight cultures were grown so that their final culture pH values ranged between 7.4 and 4.3. The results showed that stationary-phase cells taken from pH 4.3 cultures survived a subsequent 4-h pH 3.0 challenge at least 1,000 times better than pH 7.3 cells. Furthermore, induction of stationary-phase acid tolerance was not triggered until the culture pH dropped below 5.0. Clearly, there is a pH-dependent component to stationary-phase acid tolerance. Consequently, the acid-inducible stationary-phase phenomenon will be referred to as stationary-phase ATR to distinguish it from log-phase ATR.

Stationary-phase ATR requires long-term ASP synthesis. Previous work has shown that the log-phase ATR requires ASP synthesis to produce an acid-tolerant state. However, log-phase cells subjected to a nonlethal acid shock of pH 4.3 will only transiently synthesize the key ASPs for 20 to 40 min. If log-phase acid-shocked cells are challenged at a severe pH (3.3) after that time, they do not survive (7). The conclusion was that one or more of these transiently induced ASPs were essential for acid tolerance. A similar study with stationary-phase cells shown in Fig. 2A reveals that the stationary-phase ATR is very different. The results presented in this figure

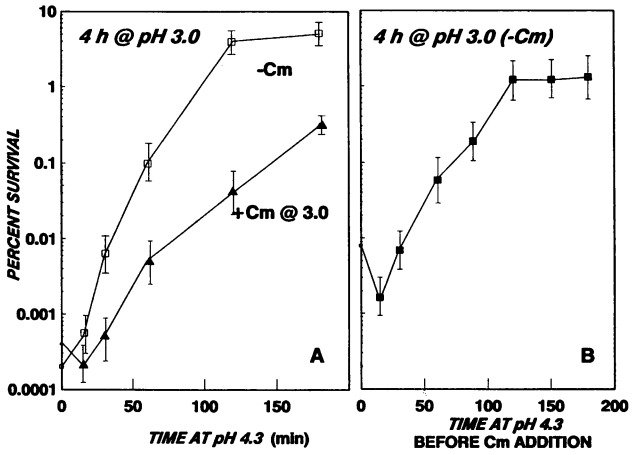

FIG. 2. Acid shock and stationary-phase ATR. (A) Cells were grown to stationary phase at pH 8.0 and resuspended as described in Materials and Methods to pH 4.3 (acid shock) for the intervals shown on the abscissa. At each time point, the acid-shocked cells were collected and resuspended at pH 3.0 either with (closed triangles) or without (open squares) chloramphenicol (40 μg/ml). An equivalent amount of solvent, ethanol, was added to the chloramphenicol-negative cultures as a control. Percent survival is shown after 4.0 h of pH 3.0 challenge. (B) pH 8 stationary-phase cultures were acid shocked at pH 4.3 as in panel A, but chloramphenicol was added at various times after the switch to pH 4.3. Total acid shock period was 180 min in all cases. After acid shock, each culture was resuspended to 2×10^8 to 5×10^8 cells per ml in pH 3.0 medium minus chloramphenicol. Percent survival is shown at 4 h after acid challenge.

(open squares) reflect what occurred when pH 8.0 stationary-phase cells were resuspended to an adaptive pH of 4.3 (acid shock) prior to severe acid challenge at pH 3.0. Acid tolerance to severe acid (pH 3.0) progressively increased with length of prior exposure to pH 4.3 acid shock. Maximum protection was afforded after 2 h of pH 4.3 exposure. The data suggested that long-term ASP synthesis was required for tolerance. Confirmation was obtained, as shown in Fig. 2B, when protein synthesis was stopped at various points during the pH 4.3 adaptation. Again, 2 h of ASP synthesis was required to achieve maximum pH 3.0 acid tolerance. The utility of chloramphenicol at acid pH was addressed earlier (6, 8). The data in Fig. 2A also reveal that maximum stationary-phase acid tolerance required continued protein synthesis at pH 3.0. The addition of chloramphenicol to cultures shifted to pH 3.0 did not eliminate low-pH-induced stationary-phase ATR but caused an overall 10- to 50-fold decrease in tolerance. This is in contrast to log-phase ATR, in which acid-shocked cells (pH 4.3) shifted to pH 3.3 will remain fully acid tolerant even without additional protein synthesis at pH 3.3 (6, 7).

ASP synthesis during stationary-phase ATR. Because the data above indicated that proteins important to stationary-phase ATR were synthesized at pH 4.3, a two-dimensional SDS-PAGE analysis of stationary-phase ASPs was conducted (Fig. 3). Cells grown overnight at pH 8 were resuspended to 2×10^8 cells per ml both at pH 4.3 and at pH 8 as a control. The proteins marked in the figure as stationary-phase ASPs are those that quantitatively increased during pH 4.3 adaptation but that did not increase at pH 8. Cells were labeled at 5 (Fig. 3A) and 60 (Fig. 3B) min. In contrast to the log-phase ATR system in which 43 ASPs were observed, only 15 ASPs were observed with the stationary-phase ATR system. Six were transient ASPs, produced at 5 but not at 60 min. We cannot

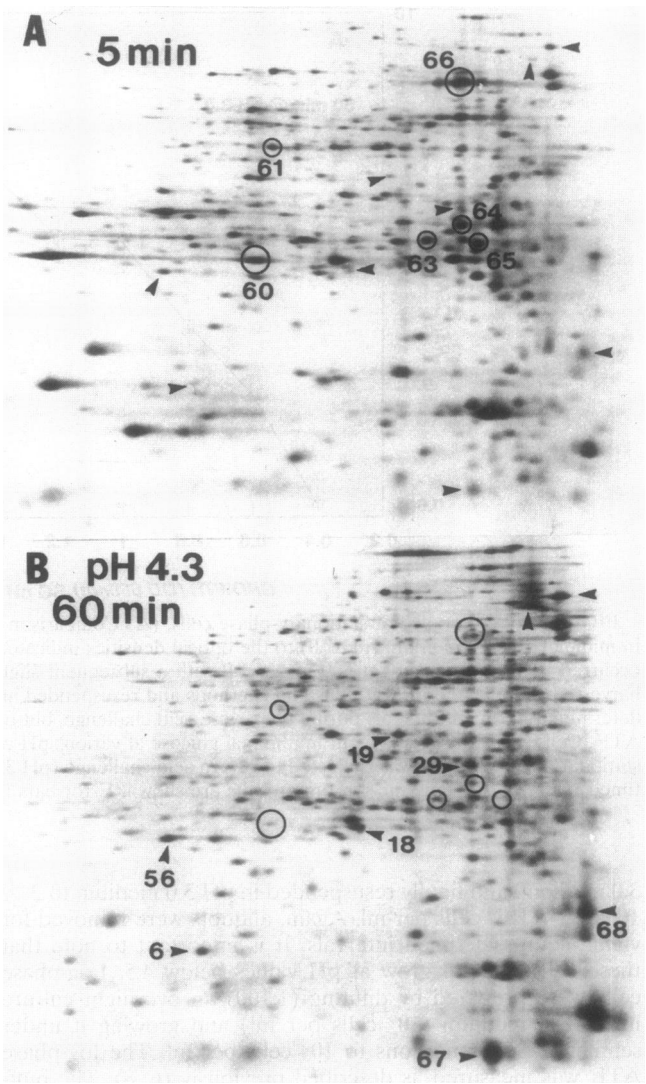

FIG. 3. Two-dimensional analysis of stationary-phase ASPs. LT2 was grown overnight at pH 8.0, and the cells were washed and resuspended to 2×10^8 cells per ml in pH 8 and pH 4.3 minimal medium. Cells were labeled for 2 min at 5 min (A) and 60 min (B). Proteins indicated are those that were induced only in the pH 4.3 cultures. Circles indicate ASPs that were induced early and transiently synthesized. Arrowheads indicate proteins induced long-term at 60 min. Numbers indicate log-phase ASPs from references 6 and 7 and stationary-phase ASPs. They are also listed in Table 1.

rule out the importance of these early ASPs to stationary-phase ATR since inhibiting protein synthesis during the first 60 min of a 3-h pH 4.3 adaptation severely reduced stationary-phase acid tolerance (data not shown). The early-phase ASPs may be needed to induce the later-phase ASPs. As noted in Table 1, several stationary-phase ASPs were previously identified as log-phase ASPs. One of these (ASP-19) is a transiently induced log-phase ASP that was expressed long-term as a stationary-phase ASP.

The role of RpoS in stationary-phase acid resistance. The growth-phase-dependent alternative sigma factor, RpoS, has been implicated in many aspects of stationary-phase physiology (23). A potential role for RpoS in pH-independent stationary-

TABLE 1. Stationary-phase ASP synthesis

Designation	Coordinates[a] (*x* vs *y*)	Regulatory overlap
ASP-56[b]	20 × 52	Log-phase ASP
ASP-60[c]	34 × 57	
ASP-6[b]	38 × 36	Log-phase ASP
ASP-61[c]	39 × 80	
ASP-19[b]	56 × 79	Log-phase ASP
ASP-18[b]	53 × 57	
ASP-62[b]	65 × 72	
ASP-63[c]	72 × 62	
ASP-29[b]	73 × 64	Log-phase ASP
ASP-66[c]	73 × 92	
ASP-64[c]	77 × 66	
ASP-65[c]	81 × 63	
ASP-66[b]	96 × 97	
ASP-67 (SIN-20[b])	76 × 18	NH_4, C
ASP-68 (IRO-40[b])	112 × 45	Iron

[a] Coordinates are based upon a standard two-dimensional SDS-PAGE analysis of *S. typhimurium* polypeptides (22).
[b] Stationary-phase ASPs induced long-term after acid shock.
[c] Stationary-phase ASPs transiently induced early (5 to 10 min) after acid shock.

phase acid tolerance was suggested for *Shigella* spp. by Gorden and Small (14) and for *S. typhimurium* by Fang et al. (4). Both studies revealed that *rpoS* mutants exhibit increased acid sensitivity. However, the studies did not explore low-pH-inducible acid tolerance. To determine whether RpoS plays a role in the acid-inducible stationary-phase ATR, we tested isogeneic *rpoS*$^+$(SL1344) or *rpoS*::Ap^r(JF2691) strains of *S. typhimurium* in our system. The results presented in Table 2 illustrate that an active *rpoS*$^+$ gene did improve total stationary-phase acid resistance 10- to 100-fold but did not change the pH-dependent stationary-phase ATR which still increased pH 3.0 survival 200- to 500-fold. The LT2 strain used for these experiments appeared to have a different *rpoS* allele compared with SL1344 on the basis of the small amount of catalase activity produced by LT2. We did find that *rpoS* has only a small effect upon log-phase ATR (Table 2).

Stationary-phase ATR is independent of log-phase ATR. Several genes associated with the log-phase ATR have been identified. Mutations in these genes, such as *atp* [Mg(II)-dependent proton-translocating ATPase] and *fur* (ferric uptake regulator), render the log-phase cell very acid sensitive because of defects in the pre- or post-acid shock stages of log-phase ATR (8, 10). Since the log-phase ATR and stationary-phase ATR appeared to be different in several ways, we examined whether *atp* (JF1923) and *fur* (JF2023) mutants would be defective, compared with LT2, in the stationary-phase ATR. The results surprisingly revealed that both strains possessed a normal low-pH-inducible stationary-phase ATR but remained defective in log-phase ATR (Table 2). Thus, neither Fur nor the major proton-translocating ATPase appears to participate in stationary-phase ATR. The results are consistent with the idea that log-phase ATR and stationary-phase ATR are distinctly different systems of acid resistance.

TABLE 2. ATR of *rpoS*, *atp*, and *fur* mutants

Strain	Genotype	ATR[a]			
		Log phase[b]		Stationary phase[c]	
		Unadapted	Adapted	Unadapted	Adapted
SL1344	*rpoS*$^+$	0.1	80	0.1	11.0
JF2691	*rpoS*::Ap	0.03	12	0.001	2.0
LT2		0.05	10	0.002	1.0
JF1923	Δ*atp*	0.005	0.003	0.0005	1.5
JF2023	*fur-1*	0.0005	0.0001	0.0002	3.0

[a] Values given are average percent survival. Initial cell densities were between 2×10^8 and 5×10^8 cells per ml. Experiments were performed in minimal glucose media.
[b] Log-phase ATR involved adapting log-phase cells for one doubling at pH 5.8 and then challenging them at pH 3.3 for 2 h.
[c] Stationary-phase ATR involved transferring stationary-phase cells (pH 8.0) to pH 4.3 for 2 h and then challenging them at pH 3.0 for 4 h.

DISCUSSION

Transient exposures to lethal levels of H^+ constitute a significant threat to prokaryotic existence. Some neutralophilic microorganisms cope with the low-pH challenges by very effective constitutive mechanisms. For example, *Mycobacterium smegmatis* will survive pH 2.5 for many hours at 100% viability (10a). Other organisms exhibit inducible responses to handle perturbations in internal pH (e.g., streptococcus [2]). The results of our studies indicate that *S. typhimurium* possesses three, possibly overlapping, strategies to enhance its acid resistance. First, there is a pH-independent general stress resistance produced by stationary-phase cells that, among other things, improves acid tolerance (reviewed by Matin [16]). This mechanism appears to be RpoS dependent. In fact, RpoS has already been implicated in the acid resistance of *Shigella flexneri*, *Escherichia coli* (14, 21), and *S. typhimurium* (4). However, it is not certain that the *rpoS*-dependent system can function in the absence of the other two *rpoS*-independent acid tolerance systems.

In contrast to the RpoS-dependent acid resistance, the two RpoS-independent acid tolerance strategies exhibited by *S. typhimurium* are both low pH inducible but differ on the basis of the physiological state of the cell at the time of acid exposure. Log-phase cells induce a two-stage system that is referred to as the log-phase ATR. It is transiently induced at a moderately low pH (<pH 4.5) but will protect cells for extended periods at severely low pH (pH 3.3). This system appears best suited to the type of rapid transitions to low pH that might be encountered by logarithmically growing cells. Stationary-phase cells are capable of mounting an *rpoS*-independent, low-pH-inducible ATR that requires a long period (2 h) of exposure to low pH for full induction. In contrast to log-phase ATR, the stationary-phase ATR system seems more appropriate for slow transitions to severe acid conditions as might be encountered by nongrowing cells. It seems logical that the cell might incorporate multiple strategies for acid protection that would reflect the basic physiological differences between stationary-phase and log-phase cells.

In another contrast with log-phase ATR, maximum acid tolerance in stationary-phase cells appears to involve continued protein synthesis during the challenge pH. This may reflect the need to compensate for increased protein turnover in stationary-phase cells and/or the contribution of the RpoS-dependent system to acid tolerance. As an alternate sigma factor, RpoS may be required for the continued synthesis of proteins involved with the stationary-phase general stress resistance phenomenon (16). Clearly, both the *rpoS*-dependent and independent stationary-phase acid tolerance systems contribute to the overall acid resistance of stationary-phase cells.

Characterization of the actual acid-protective mechanisms that compose the low-pH-inducible ATR systems will be aided by the identification of the various low-pH-induced polypeptides. Several classes of ASPs have been identified. They

include log-phase-specific ASPs, stationary-phase-specific ASPs, and ASPs produced by both log-phase and stationary-phase cells. It is not known which of the ASPs are directly associated with acid protection. However, among the log-phase ASPs, it is apparent that one or more of the 14 in the transiently induced subset are the key to acid tolerance. It is intriguing that one of the transiently induced log-phase ASPs was also shown to be a long-term stationary-phase ASP. The set of ASPs common to both log-phase and stationary-phase cells may also prove interesting in terms of a role common to both ATR systems. Potential mechanisms of acid tolerance include enhanced pH homeostasis, repair or prevention of acid damage to macromolecules, and replacement of acid-sensitive cellular components with acid-resistant homologs. Evidence for enhanced pH homeostasis has been presented for log-phase ATR (9).

The potential role of acid resistance in virulence is an underlying question for these studies. It is predicted that gastric acidity forms a protective barrier against oral-route infection (3, 12). In addition, pathogens such as *S. typhimurium* can encounter low-pH environments in the intestine and phagolysosome environments (5, 18). Although it may be intuitively obvious, a direct link between acid resistance and virulence remains to be established for *S. typhimurium*. However, preliminary results correlating acid tolerance and virulence are promising (11, 25). Work by Gorden and Small (14) suggests that the low infective dose required for oral administration of *S. flexneri* is due to the high degree of stationary-phase acid resistance displayed by this organism. Ultimately, the characterization of genes involved with acid resistance will contribute to our general knowledge of procaryotic stress management strategies and their roles in virulence.

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